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Relaxant activity in rat aorta and trachea, conversion to a muscarinic receptor antagonist and structure–activity relationships of new K_{ATP} activating 6-varied benzopyrans

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Abstract

To characterize ATP-sensitive channels (K_{ATP} channels) benzopyrans with different substituents at position 6 were synthesized as new K_{ATP}-activators. Their relaxant potencies were determined in rat aorta and trachea. In aorta, pEC_{50}-values ($-\log M$) ranged from 7.37 to 5.43; in trachea, pEC_{50}-values were 0.3 to 0.8 log units lower. Functional data were compared with binding data obtained in calf tracheal cells using the cyanoguanidine [^{3}H]P1075 (*N*-cyano-*N*′-1,1-dimethyl[2,3(*n*)-^{3}H]propyl)-*N*^{11}-(3-pyridinyl)guanidine) as radioligand. A high correlation ($r = 0.96$) between pEC_{50}- and pK_D-values indicated that tracheal relaxation produced by benzopyrans is mediated via K_{ATP} channels without signal amplification. The permanently charged trimethylammonium derivative designed as a probe for the membrane site of action completely lost its affinity for K_{ATP} channels, but converted to an antagonist for muscarinic acetylcholine receptors ($pK_B = 6.12 \pm 0.10$), as confirmed in radioligand binding studies ($pK_D = 5.77 \pm 0.04$). Structure–activity analyses revealed that the 6-substituent influences biological activity by a direct receptor interaction of its own and not indirectly by withdrawing electrons from the benzopyran nucleus. The variance of the biological activity is primarily determined by electrostatic properties, but desolvation energies additionally contribute.

Keywords: K_{ATP} channel; K^+ channel activator; Benzopyran; Smooth muscle relaxation; QSAR (quantitative structure–activity relationships) analysis; Muscarinic receptor antagonism

1. Introduction

During the last two decades, the treatment of hypertension has undergone a remarkable revolution with the introduction of several classes of drugs like calcium antagonists and angiotensin converting enzyme inhibitors. More recently, a new class of compounds has been detected which lowers blood pressure by a novel mechanism of action, involving an increase in the outward movement of K^+ ions through K^+ channels in the membranes of vascular smooth muscle cells, finally resulting in the relaxation of smooth muscle. These compounds modulate the activity of K_{ATP} channels which are composed of a small inwardly rectifying K^+ channel subunit plus a sulfonylurea receptor (SUR) that carries the binding sites for K_{ATP} channel agonists and antagonists (Aguilar-Bryan et al., 1998; Hambrock et al., 1998; Schwanstecher et al., 1998).

Among these compounds, termed K^+ channel activators, cromakalim was the first drug shown to act exclusively by this mechanism (Hamilton et al., 1986). Chemically, cromakalim is a benzopyran, the best investigated subgroup among K^+ channel activators. Structure–activity studies of the benzopyrans mainly focus on variations in the 4-position of the benzopyran nucleus whereas systematic investigations of variations in the 6-position are very limited (Bergmann and Gericke, 1990; Gericke et al., 1991; Ishizawa et al., 1993; Ohta et al., 1994).

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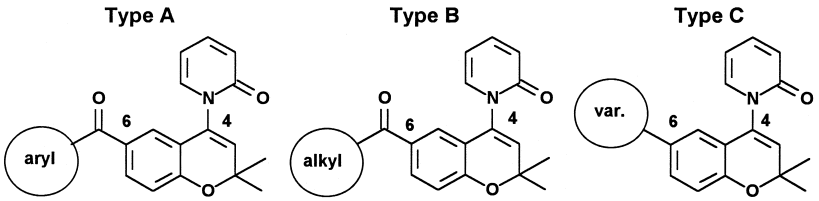

Scheme 1.

As a lead for benzopyrans systematically derivatized in the 6-position we selected bimakalim, which is well characterized as a K^+ channel activator (Gericke et al., 1991) due to its high activity (Mannhold et al., 1996, 1999), its high affinity for K_{ATP} channels of cardiomyocytes (Lemoine et al., 1998) and of smooth muscle cells of the coronary artery (Lemoine et al., 1996) and its lack of chirality. Three types of new benzopyrans were used as depicted in Scheme 1.

In the present paper, we describe the relaxant properties of these compounds in aortic and tracheal smooth muscle of the rat and define structure–activity relationships with special regard to the question whether the 6-substituent influences biological activity either by modulating the electronic properties of the aromatic part of the benzopyran nucleus or by a receptor interaction of its own.

2. Materials and methods

2.1. Isolated tissues

2.1.1. Tissue preparation

Male Wistar rats (body weight 300–400 g) were anesthetized with ether and the chest was opened. The trachea and aorta were removed and washed free of blood with modified Krebs solution containing (mM) 90.0 NaCl, 29.0 $NaHCO_3$, 5.0 KCl, 1.0 Na_2HPO_4, 0.5 $MgSO_4$, 0.04 EDTA, 2.25 $CaCl_2$ equilibrated with carbogen (95% O_2, 5% CO_2). The water was deionized and double distilled in glass. After the removal of connective tissue, fat and endothelium, aortas were cut into rings of 3 mm width and mounted in pairs in a modified Blinks apparatus equipped with 7-ml organ baths (Blinks, 1965) without further manipulations. Tracheas were opened on the opposite side of the muscle layer, tied on one end to a thread (Flexafil 6/0, J. Pfrimmer, Erlangen) and also set up in the Blinks apparatus. Each strip was attached to a strain-gauge transducer connected via amplifiers to polygraphs (Watanabe V) for isometric force measurement. In the organ baths, the modified Krebs solution was supplemented with (mM) pyruvate 5, fumarate 5, L-glutamate 5, glucose 10, and ascorbate 0.2. Resting force was adjusted to 5 mN (aorta) or 10 mN (trachea) at the beginning of an experiment and remained unchanged throughout the experiment. Experiments were carried out at 32.5°C.

2.1.2. Concentration–effect curves

At the beginning of each experiment, the modified Krebs solution was supplemented with 5 mM fumarate, 5 mM pyruvate, 5 mM L-glutamate and 10 mM glucose and the tissues were allowed to equilibrate in the bath solution for 2 h followed by two successive test contractures at 60-min time intervals. In aortic rings K^+-contractures were induced by replacing 20 mM NaCl by KCl in the Krebs-solution described above, resulting in a final concentration of 25 mM KCl. In tracheal strips, test contractures were induced with 0.6 μM carbachol. A third contracture was induced to record a cumulative concentration–effect curve for a K^+ channel activator. In each experiment, eight aortic rings and eight tracheal strips from two animals were investigated simultaneously. Two of the eight preparations from each animal served as controls to record the spontaneous changes in tension. Effects (E, %) are expressed as percentages (Δ, %) of basal and maximum force measured before and after induction of contractures, respectively. Concentration–effect curves for the test compounds were constructed with individual effects (E, %) and pEC_{50}-values were measured by linear extrapolation from half-maximal effect levels to drug concentrations. Maximum effects were determined by considering basal force before induction of the contracture as 100% relaxation. Means ± standard errors of the mean (S.E.M.) were calculated for $n = 4–6$ experiments.

2.1.3. Estimation of the dissociation constants for blockade

In order to evaluate if AE 51 acts as an antagonist at muscarinic acetylcholine receptors, five cumulative concentration–effect curves for carbachol were determined, one in the absence and four in the presence of increasing concentrations of AE 51. Concentration ratios (CR) for the agonist were measured at 50% effect levels as the ratio of carbachol concentrations in the absence and presence of AE 51. CR-values depend on blocker concentrations [B] according to Arunlakshana and Schild (1959)

$$\log(CR - 1) = m \cdot \log[B] - \log K_B \quad (1)$$

where K_B is the equilibrium dissociation constants of AE 51 and m is the slope of the regression line. Data were analyzed by linear regression analysis. When m was not significantly different from unity, the mean pK_B (−log, M) was calculated as $pK_B = \log(CR - 1) - \log[B]$ for each

of the tested antagonist concentrations (Lemoine and Kaumann, 1983).

2.2. Isolation of tracheal smooth muscle cells

Cells were isolated by enzymatic disaggregation as published (Lemoine et al., 1989, 1997) with some modifications (Teschemacher et al., 1998). In brief, tracheal strips were freed from cartilage, mucosa and connective tissue. Cylindrical strips of less than 0.8 mm width and 5–10 mm length were cut along the natural direction of the ring muscle fibers. The strips were incubated in an isotonic potassium/methanesulfonate solution containing 130 mM KOH, 20 mM taurine, 5 mM pyruvate, 5 mM creatine, adjusted with 10 mM HEPES and methanesulfonic acid to pH 7.4 and supplemented with 70 mg/100 ml collagenase D (Boehringer, Mannheim), 10 mg/100 ml pronase E (Serva, Heidelberg) and 0.1 mM $CaCl_2$. Tracheal strips were incubated in three Erlenmeyer flasks (100 ml), each with 10 ml of collagenase medium at 37°C, on a magnetic stirrer stirring at 60 rpm. Every 30 min, the cell-rich supernatant was harvested with pasteur pipettes. The cells were spun down at 200 g for 10 min and washed three times with potassium/methanesulfonate buffer. After seven to eight incubation periods, the muscle strips were completely disaggregated. More than 2/3 of the cells were elongated and fusiform and less than 1/3 had a rounded appearance, as observed by phase-contrast microscopy.

2.3. Radioligand binding

For radiolabelling of K_{ATP} channels we used [^{3}H]P1075 (*N*-cyano-*N'*-1,1-dimethyl[2,3(*n*)-^{3}H]propyl)-*N*^{11}-(3-pyridinyl)guanidine) as radioligand (Manley et al., 1993; Quast et al., 1993). The specific activity of [^{3}H]P1075 was 108 Ci/mmol. The incubation was performed in a Ca^{2+}-free cell culture medium (minimum essential medium eagle supplemented, MEMS) at 37°C. The radioligand was incubated for 60 min, which guarantees complete equilibration with K_{ATP} channels of tracheal smooth muscle cells (Lemoine et al., 1997). The binding observed in the presence of 1 μM P1075 was regarded as non-specific. For radiolabelling of muscarinic acetylcholine receptors, we used [^{3}H]quinuclidinyl[phenyl-4-^{3}H]benzilate (QNB), which had a specific activity of 50 Ci/mmol. With this ligand non-specific binding was examined in the presence of 1 μM atropine.

Bound radioligand was separated from free radioligand by rapid vacuum filtration through Whatman GF/A glass fiber filters pretreated with ice-cold polyethyleneglycole-buffer (10% PEG, 10 mM Tris, 10 mM $MgCl_2$, pH 7.4). The filters were rapidly washed four times with 5 ml ice-cold buffer (10 mM Tris, 5 mM $MgCl_2$, pH 8.0) and counted in a scintillation-counter (Packard 1500) with an efficiency of 50% using Ultima Gold as scintillator (Packard, USA). Competition binding experiments were performed in the presence of a constant concentration of the radioligand [L*] (1 nM [^{3}H]P1075 for K_{ATP} channels, 0.4 nM [^{3}H]QNB for muscarinic acetylcholine receptors) and increasing concentrations of competing ligands [L]. Displacement curves were analyzed by non-linear regression as reported (Lemoine, 1992; Lemoine et al., 1985) according to the following equation

$$B_s([L]) = B_o - B_o \frac{[L]}{[L] + K_D(1 + [L^*]/K_L^*)} \quad (2)$$

where B_o and B_s([L]) represent the specific binding of L* to K_{ATP} channels in the absence and presence of L, K_L^* denotes the dissociation constant of the respective radioligand L* estimated independently in saturation binding experiments and K_D means the equilibrium dissociation constant of the test compound L. Experimental data were analyzed after transformation of data to obtain homoscedasticity (Ehle et al., 1985), which resulted in reliable estimates of parameters (B_o, B_{ns} and pK_D) and asymptotic standard deviations (A.S.D.). Data were fitted to the hyperbola defined by Eq. (2) by non-linear regression with the use of SAS software package STAT. Data points presented in the figures are means ± S.E.M.

2.4. Modelling techniques

The molecular modelling studies were carried out on Silicon Graphics Indigo2 workstations with SYBYL6.3 (Tripos associates, St. Louis, MO, USA). The conformational behavior of all compounds was investigated using molecular mechanics as well as quantum chemical methods. The charge distribution (molecular electrostatic potential) was calculated with a 3–21 G* basis set. Energies of the lowest unoccupied molecular orbital (LUMO), which is located at the benzene ring (LUMO(benz)), were extracted from a semiempirically derived wavefunction (AM1-method), using the software package SPARTAN 4.1 (Wavefunction, Irvine, USA). Solvation energies were generated applying a semianalytical approach, which is implemented in the program PrGen2.0 (Biographics Laboratory, Basel, CH).

2.5. Drugs and materials

The radioligands [^{3}H]P1075 (108 Ci/mmol = 4.00 TBq/mmol) and ^{3}H-QNB (quinuclidinyl[phenyl-4-^{3}H] benzilate, 50 Ci/mmol = 1.85 TBq/mmol) were from the Radiochemical Center, Amersham, UK. Glibenclamide was from Hoechst Marion Roussell (Frankfurt). K^+ channel openers were synthesized by A. Derix and H. Weber (Ewertz et al., 1997; Derix, 1998) or were from E. Merck (Darmstadt). K^+ channel openers were dissolved in 10% dimethyl sulfoxide (DMSO). The DMSO concentration in the organ bath was kept lower than 1% (v/v) and did not influence tissue responsiveness. MEMS 10-171-22 was from ICN (Costa Mesa, USA); other chemicals were from local commercial sources.

3. Results

3.1. Biological activity in isolated aorta and trachea

To quantify the relaxant potencies of the different K^+ channel activators we recorded cumulative concentration–effect curves for the test compounds in isolated aortic rings and tracheal strips of the rat. Under control conditions, contractures induced with 25 mM K^+ in aortic rings (Fig. 1, 1st trace) and with 0.6 μM carbachol in tracheal strips (Fig. 1, 3rd trace) were constant for 5 to 10 h. Contractures of tracheal strips elicited by 25 mM K^+ were not stable for the time period needed. The test compounds relaxed smooth muscle preparations dose dependently. As a typical example, cumulative concentration–effect curves for AE 30 are shown as original recordings both in rat aorta (Fig. 1, 2nd trace) and in rat trachea (Fig. 1, 4th trace). AE 30 was more potent in aorta than in trachea (compare the effects of 2 and 6 μM AE 30). Fig. 2 shows the corresponding concentration–effect curves of AE 30 for relaxation of aortic and tracheal smooth muscle. Mean pEC_{50}-values ($-\log, M$) and S.E.M. for the relaxation produced by AE 30 were calculated as 6.17 ± 0.05 for the aorta and 5.64 ±

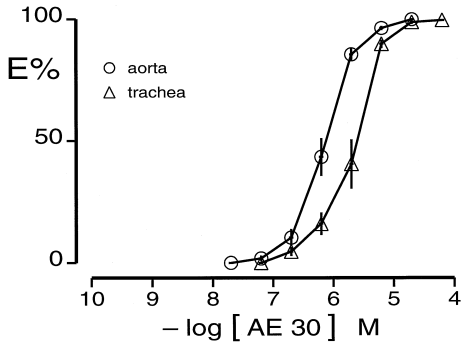

Fig. 2. Concentration–effect curves for compound AE 30 in aortic rings (circles) and tracheal strips (triangles). Points and bars represent means ± S.E.M. of $n = 6$ aortic rings and $n = 5$ tracheal strips. pEC_{50}-values for relaxation were determined as 6.17 ± 0.05 (aorta) and 5.64 ± 0.04 (trachea).

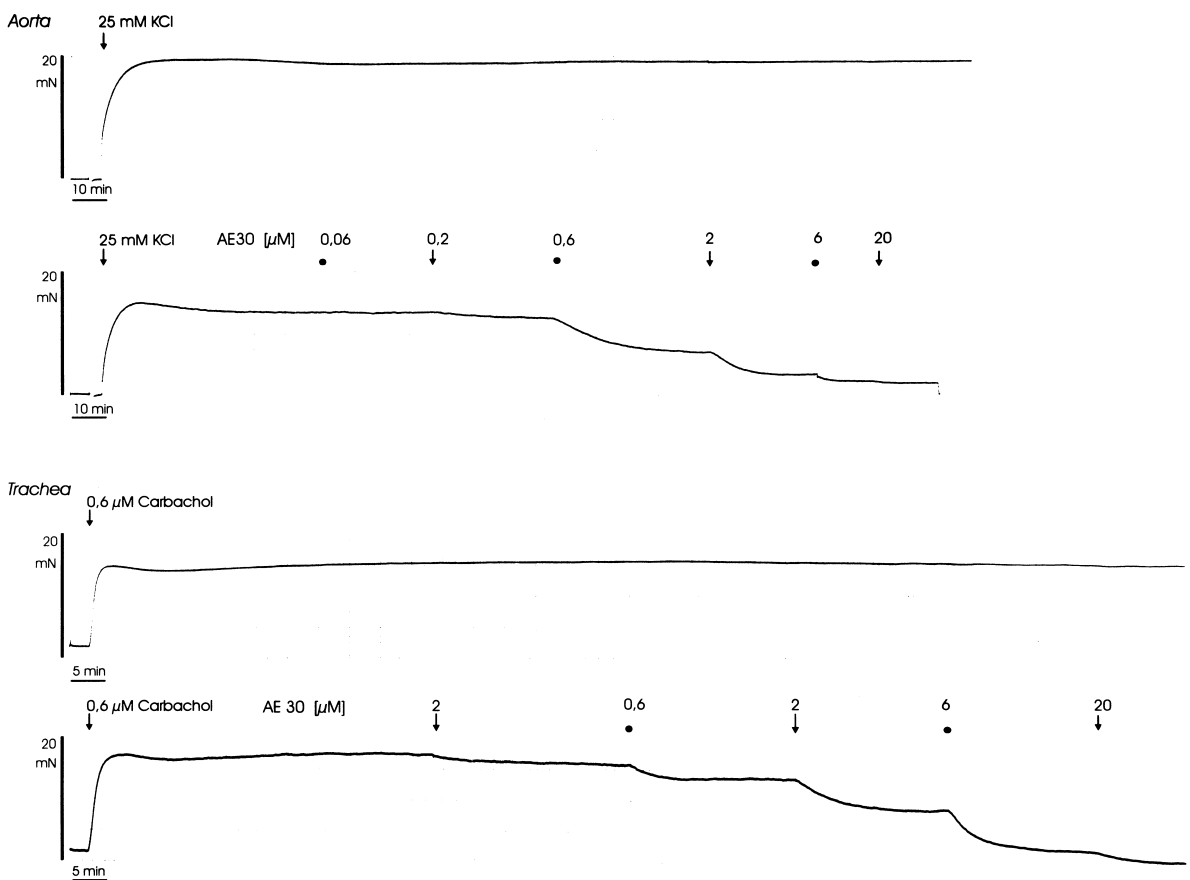

Fig. 1. Original recording of an experiment with compound AE 30. Top panel: Two aortic rings from the same animal were mounted in different organ baths, one served as control (1st trace) and the other was used to test AE 30 (2nd trace). Basal tone of aortic rings was adjusted to 5 mN and remained stable for 2 h (not shown) before a contracture was induced with 25 mM KCl. After the contracture reached equilibrium, increasing concentrations of AE 30 were added to the second aortic ring (2nd trace) at the indicated time points. Bottom panel: Two tracheal strips from the same animal were mounted in different organ baths, one served as control (3rd trace) and the other was used to test AE 30 (4th trace). Basal tone of a tracheal ring was adjusted to 10 mN and remained stable for 2 h (not shown) before a contracture was induced with 0.6 μM carbachol. After the contracture reached equilibrium, increasing concentrations of AE 30 were added to the second aortic ring (4th panel) at the indicated time points. Note that the time scales in the top and bottom panels are different, indicating a faster drug access to trachea than to aorta.

0.04 for the trachea (compare Table 1), indicating that AE 30 was approximately three times more potent in aorta.

In order to attribute the smooth muscle relaxation caused by the new compounds to the activation of K_{ATP} channels, the reversibility of the relaxant effects was tested by application of the K^+ channel antagonist glibenclamide (data not shown). In all cases, the relaxant effects were effectively antagonized with 2, 6, and 20 μM glibenclamide added cumulatively to the organ baths after the relaxation caused by the test compounds had reached a maximum.

The relative potencies of the new benzopyrans in the two test systems were measured in four to six experiments (Table 1). In rat aorta, pEC_{50} values differed by 2.4 log units and ranged from 7.84 ($-\log M$) for the bromo-derivative (EMD 55491) with the highest potency to 5.43 ($-\log M$) for the unsubstituted lead (AE 9) with the lowest potency. In rat trachea, the activity spectrum differed by 2.5 log units; the highest potency was again found for EMD 55491 ($pEC_{50} = 7.53 \pm 0.11$) and the lowest for AE 9 ($pEC_{50} = 5.05 \pm 0.08$). Comparison of the potency of the compounds to cause relaxation in aortic vs. tracheal smooth muscle by linear regression revealed a slope near 1, indicating a pronounced similarity of K_{ATP} channels in both tissues:

$$\log 1/C_{aorta} = 0.924(\pm 0.17)\log 1/C_{trachea} + 1.012(\pm 1.07) \quad (3)$$

$n = 24$; $r = 0.921$; $s = 0.23$; $F = 123$

On average, the compounds were about 0.5 log units more potent in aortic rings than in tracheal strips.

3.2. Binding-function correlations

To prove whether the relaxant properties of the new benzopyrans are mediated via K_{ATP} channels, we performed a binding study with enzymatically disaggregated tracheal cells of the calf. Recently, we published (Lemoine et al., 1997) that the cyanoguanidine [^{3}H]P1075 is a suitable radioligand for K_{ATP} channels of tracheal smooth muscle cells and is characterized by high affinity ($pK_D = 8.62 \pm 0.09$) and low non-specific binding. For the binding study, the following selection of test compounds was made: bimakalim (R^6 = CN) as reference, AE 9 (R^6 = −H) as the unsubstituted lead to quantify the impact of 6-subs-

Table 1
Relaxant potencies of benzopyrans with different substituents at position 6 in rat aorta and trachea
Relaxant potencies are given as pEC_{50}-values ($-\log M$) and E_{max}-values (%) refer to basal tone set to 100% relaxation. S.E.M. values are standard errors of the mean for n independent experiments.

Compound	R^6	Aorta			Trachea		
		pEC_{50} (±S.E.M.) $-\log M$	n	E_{max} (±S.E.M.) (%)	pEC_{50} (±S.E.M.) $-\log M$	n	E_{max} (±S.E.M.) (%)
Type A compounds							
AE 09	H	5.43 (±0.06)	6	96 (±1)	5.05 (±0.08)	6	92 (±1)
AE 20	Benzoyl	6.61 (±0.07)	6	101 (±1)	6.21 (±0.07)	6	104 (±5)
AE 22	2-Furoyl	6.49 (±0.05)	6	101 (±1)	5.92 (±0.04)	4	99 (±5)
AE 23	2-Thenoyl	6.40 (±0.10)	5	100 (±0)	5.84 (±0.08)	6	100 (±0)
AE 21	4-Hydroxybenzoyl	6.65 (±0.03)	4	100 (±0)	5.87 (±0.12)	4	101 (±4)
AE 25	4-Methoxybenzoyl	6.15 (±0.13)	4	94 (±5)	5.72 (±0.03)	4	111 (±3)
AE 26	4-Nitrobenzoyl	6.20 (±0.13)	6	98 (±3)	6.09 (±0.11)	6	102 (±2)
AE 33	2-Methylbenzoyl	6.83 (±0.09)	5	98 (±3)	6.25 (±0.08)	4	98 (±2)
AE 34	2-Trifluoro-methylbenzoyl	6.76 (±0.14)	4	99 (±3)	5.78 (±0.16)	4	98 (±3)
AE 30	2-Nitrobenzoyl	6.17 (±0.05)	6	97 (±1)	5.64 (±0.04)	5	100 (±0)
AE 29	2-Fluorobenzoyl	7.08 (±0.13)	6	96 (±5)	6.34 (±0.12)	6	99 (±1)
AE 49	2,6-Difluorobenzoyl	6.97 (±0.09)	5	99 (±1)	5.99 (±0.06)	5	102 (±1)
Type B compounds							
AE 44	Formyl	6.91 (±0.05)	6	100 (±1)	6.14 (±0.03)	5	101 (±2)
AE 11	Acetyl	7.37 (±0.05)	6	101 (±1)	6.65 (±0.01)	6	100 (±0)
AE 15	Propionyl	6.63 (±0.10)	6	100 (±0)	5.85 (±0.07)	6	99 (±1)
AE 18	Cyclohexyl-carbonyl	6.33 (±0.15)	4	86 (±6)	6.03 (±0.10)	4	100 (±0)
Type C compounds							
Bimakalim	Cyano	7.67 (±0.10)	5	99 (±2)	7.10 (±0.08)	6	101 (±1)
AE 47	2,2-Dicyanoethenyl	6.95 (±0.08)	6	96 (±4)	6.30 (±0.07)	5	99 (±2)
EMD 55491	Bromo	7.84 (±0.08)	5	98 (±1)	7.53 (±0.13)	6	109 (±4)
EMD 60893	Trifluoromethyl	7.61 (±0.12)	5	101 (±2)	7.37 (±0.05)	6	100 (±1)
AE 45	Aminohydroxy-iminomethyl	5.99 (±0.13)	6	99 (±1)	5.51 (±0.11)	6	106 (±5)
EMD 54208	Thioamido	6.21 (±0.12)	6	101 (±2)	5.96 (±0.06)	5	101 (±1)
EMD 53704	4-Pyridyl	6.68 (±0.10)	6	106 (±5)	6.32 (±0.12)	6	115 (±5)
AE 48	2,5-Dimethyl-1-pyrrolyl	7.28 (±0.08)	5	98 (±3)	6.78 (±0.06)	4	101 (±1)

titution, AE 11 (R^6 = acetyl), AE 15 (R^6 = propionyl) and AE 20 (R^6 = benzoyl) as a series with increasing size and lipophilicity, and the bromo-derivative (EMD 66491) to test if its electronic properties favor high affinity compared to that of the reference. [^{3}H]P1075 binding could be completely displaced by all test compounds in competition binding experiments (not shown). In all experiments, binding curves followed a simple hyperbola (Eq. (2)) and non-specific binding did not exceed 25%. Dissociation constants of the test compounds estimated by non-linear regression are listed in Table 2. As found in the relaxation study, the Br- (EMD 55491) and the H-derivative (AE 09) exhibited the highest ($pK_D = 7.79 \pm 0.04$) and the lowest affinity ($pK_D = 5.52 \pm 0.09$), respectively. The affinity decreased with increasing size of the R^6-substituent (compare AE 11, AE 15 and AE 20) and the bromo-derivative exhibited a two-fold higher affinity than bimakalim.

To determine whether the binding affinities of the tested compounds measured in calf tracheal cells correspond to their potencies for relaxation of rat tracheal strips, the correlation of pK_D-data for binding and pEC_{50}-data for relaxation was calculated. The plot in Fig. 3 and the following regression equation showed there to be a high interrelation between binding and functional data:

$$pEC_{50} = 0.79(\pm 0.23)pK_D + 0.99(\pm 1.59) \quad (4)$$

$n = 8$; $r = 0.96$; $s = 0.24$; $F = 67.8$

This correlation indicates that smooth muscle relaxation induced by the new benzopyrans is due to binding to K_{ATP} channels. The moderately higher affinity of the compounds in binding studies (tracheal cells of the calf) as compared to their relaxant potency in functional experiments (tracheal strips of the rat) might reflect species differences.

3.3. Characterization of the anti-muscarinic activity of AE 51

The R^6-substituent of the compound AE 51, which was designed to discriminate between intra- and extracellular access of benzopyrans to their binding sites on K_{ATP} channels, is a permanently charged trimethylammonium group with strong electronegative properties. First experiments with AE 51 in aortic rings and tracheal strips (Fig. 4) revealed a marked difference in its relaxant potency. While tracheal tension was potently reduced with micromolar concentrations of AE 51, aortic tension was completely resistant to relaxation. As all other compounds (see Table 1) did not show marked difference in potencies in aorta and trachea, which argues against a high selectivity of AE 51 for tracheal K_{ATP} channels, we considered an alternative hypothesis related to the different stimulus chosen for precontraction of aortic (25 mM K^+) and tracheal (0.6 μM carbachol) smooth muscle.

As an alternative mechanism we hypothesized that AE 51 might directly antagonize carbachol at muscarinic acetylcholine receptors. Consequently, increasing the occupancy of muscarinic receptors by the agonist should reduce the relaxant potency of AE 51. However, high concentrations of carbachol could induce a different spectrum of intracellular events due to a shift of signal transduction by occupation of different muscarinic receptor subtypes. Therefore, we chose a different method to increase muscarinic receptor occupancy and combined a high concentration of agonist with an appropriate concentration of an antagonist, resulting in the same degree of receptor occupancy by the agonist. We used 100 nM atropine (equivalent to approximately 100 K_D) and 60 μM carbachol,

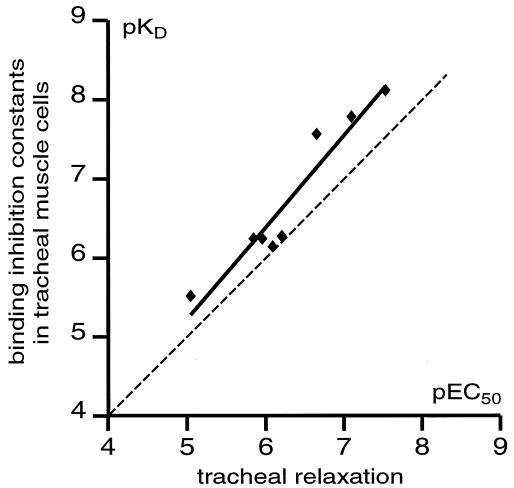

Fig. 3. Correlation of pK_D-values representing binding affinity with pEC_{50}-values for relaxation of rat tracheal strips of a selection of test compounds. From left to right, data points represent AE 09, AE 15, EMD 54 208, AE 26, AE 20, AE 11, bimakalim and EMD 55491. Binding was measured as competition binding with [^{3}H]P1075 in enzymatically disaggregated tracheal smooth muscle cells of the calf. Values for pEC_{50} and pK_D are listed in Table 1 and Table 2, respectively. The dotted line represents the hypothetical identity of pK_D and pEC_{50}. The regression line with a slope of 1.16 ± 0.08 shows a high correlation ($r = 0.96$) between binding and functional data. The deviation of the regression line from the dotted line reflects a higher potency of compounds for binding than for relaxation, possibly indicating species differences between tracheal smooth muscle of calf and rat.

Table 2
Binding inhibition constants of a selection of some K^+ channel activators for the [^{3}H]P1075-labelled binding site in tracheal smooth muscle cells
pK_D values were determined in competition binding experiments with [^{3}H]P1075 as radioligand (experiments not shown). Competition binding curves were fitted by non-linear regression analysis according to Eq. (2). A.S.D. values reflect asymptotic standard deviations estimated by regressions analysis.

Compound	Substituent	$pK_D \pm$A.S.D, $-\log, M$
Bimakalim	CN	7.79 ± 0.04
AE 9	H	5.52 ± 0.09
AE 11	Acetyl	7.57 ± 0.17
AE 15	Propionyl	6.25 ± 0.16
AE 20	Benzoyl	6.28 ± 0.25
AE 26	4-Nitrobenzoyl	6.15 ± 0.18
EMD 54208	Thioamido	6.25 ± 0.07
EMD 55491	Bromo	8.12 ± 0.06

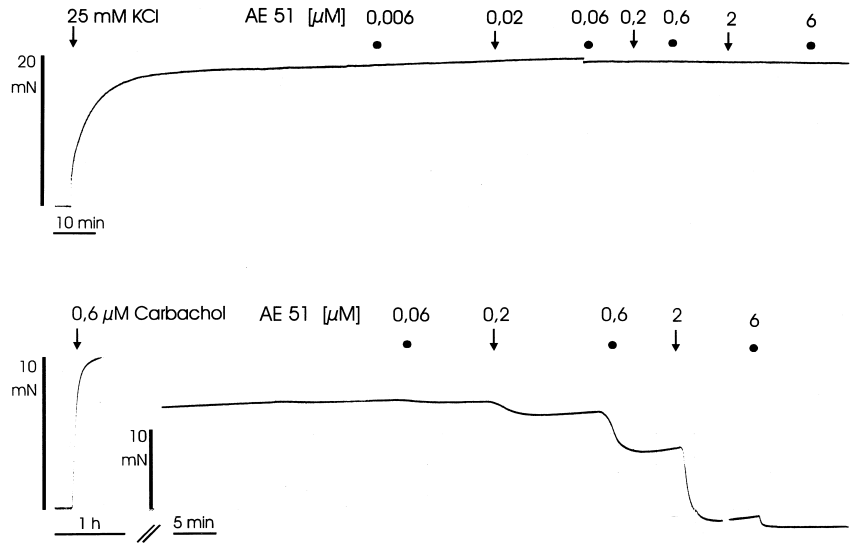

Fig. 4. Original recording of an experiment with the trimethylammonium derivative AE 51. Top trace: Basal tone of an aortic ring was adjusted to 5 mN and remained stable for 2 h (not shown) before a contracture was induced with 25 mM KCl. After the contracture reached equilibrium, increasing concentrations of AE 51 were added at the indicated time points. Aortic tone was resistant to relaxation induced by AE 51. Bottom trace: Basal tone of a tracheal ring was adjusted to 10 mN and remained stable for 2 h (not shown) before a contracture was induced with 0.6 μM carbachol. After 1 h, the registration system was reamplified and the time resolution was readjusted (see calibration bars). Concentrations of AE 51 were increased from 0.06 to 6 μM and induced a dose-dependent and complete relaxation of the tracheal strip.

which exceeded the standard concentration by a factor of 100, and applied this agonist/antagonist combination to precontract tracheal strips to achieve a similar height of tracheal tone. Under this condition, the reanalysis of the tracheal relaxation produced by AE 51 resulted in a marked loss of relaxant potency (Fig. 5): micromolar concentrations of AE 51 were completely ineffective and high concentrations, which were maximally effective under standard conditions, exhibited only threshold effects.

From this experiment (Fig. 5), it appeared likely that AE 51 acts as antagonist for muscarinic acetylcholine

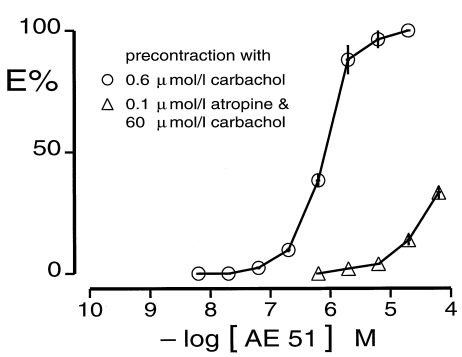

Fig. 5. Tracheal relaxation elicited by AE 51 is dependent on a different occupancy of muscarinic receptors. Standard conditions (circles, 0.6 μM carbachol) for precontraction of tracheal strips were compared to a combination (triangles) of an agonist (60 μM carbachol) with the appropriate antagonist (0.1 μM atropine), resulting in the same receptor occupancy by the agonist alone but a 100-fold higher receptor occupancy by the combination of agonist and antagonist. The potency of AE 51 for relaxation was markedly reduced after precontraction with 60 μM carbachol in the presence of 0.1 μM atropine, indicating an anti-muscarinergic effect of AE 51.

receptors. Thus, a further series of experiments was performed to determine the equilibrium dissociation constant of AE 51 for muscarinic acetylcholine receptors (Fig. 6). Concentration–effect curves for carbachol were determined in the absence and presence of AE 51. From the AE 51-induced shift of the concentration–effect curves to the right (i.e., higher carbachol concentrations), CR were determined as log(CR − 1)-values and related to AE 51 concentration ($-\log, M$). The linear regression analysis of data according to Eq. (1) (Arunlakshana and Schild, 1959) yielded a slope of approximately 1.00 (see Fig. 6, bottom panel) and an equilibrium dissociation constant K_B ($-\log, M$) of 6.12 ± 0.12. These experiments and the observation that AE 51 did not exhibit any stimulant effects characterize the compound as a pure antagonist of muscarinic acetylcholine receptors with micromolar affinity.

To exclude the possibility that AE 51 acted as antagonist in aortic smooth muscle, a possibility which was not analyzed in the experiment of Fig. 4, we designed an experiment with aortic rings (not shown) in which AE 51 was regarded as a putative antagonist of the agonistic effects of another compound of the series (AE 11). Putative antagonistic properties of AE 51 would result in a rightward shift of the concentration–response curve for AE 11. However, under both conditions (i.e., in the absence and presence of AE 51) identical concentration–response curves for AE 11 were measured, thereby excluding any K_{ATP}-antagonistic activity of AE 51 in aortic smooth muscle.

To confirm the characterization of AE 51 as an antagonist with a micromolar K_B for muscarinic acetylcholine receptors without any affinity for K_{ATP} channels we per-

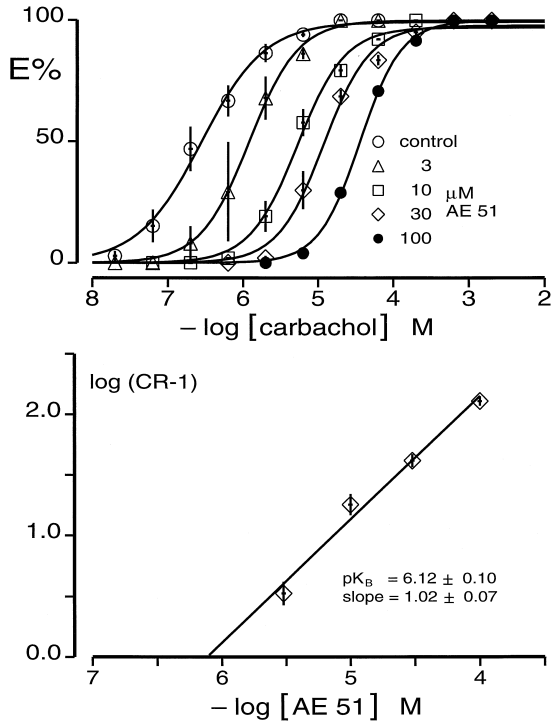

Fig. 6. Determination of the equilibrium dissociation constant of AE 51 as antagonist of muscarinic receptors in rat tracheas. Upper panel: Concentration–effect curves for carbachol were determined in absence and presence of different concentrations of AE 51. AE 51 was preincubated for 60 min. Lower panel: Schild-plot relating AE 51 concentrations to the CR of carbachol determined as the difference between pEC_{50}-values in the absence and presence of antagonist (AE 51). Linear regression of data resulted in a slope not significantly different from one and a dissociation constant K_B ($-\log$, M) of 6.12 ± 0.10.

formed binding experiments with tracheal smooth muscle cells of the calf. Muscarinic acetylcholine receptors were labelled with [^{3}H]QNB and K_{ATP} channels with [^{3}H]P1075. Whereas [^{3}H]QNB was effectively displaced by increasing concentrations of AE 51, the binding of [^{3}H]P1075 was completely resistant to AE 51 (Fig. 7). Non-linear regression analysis according to Eq. (2) using the dissociation constant (10.11 ± 0.05) for [^{3}H]QNB determined in tracheal smooth muscle cells by saturation binding (Teschemacher et al., 1998), yielded a dissociation constant ($-\log$, M) for AE 51 of 5.77 ± 0.04. Binding data confirmed the results of functional experiments, showing micromolar affinity of AE 51 for muscarinic acetylcholine receptors and the complete loss of affinity for K_{ATP} channels.

3.4. Analysis of structure–activity relationships

3.4.1. Qualitative structure–activity relationships

The *type A compounds* comprised aryl-keto-benzopyrans with moderate potency. Exchange of benzoyl by furoyl or thenoyl decreased potency. Also *para*-substitution was detrimental with following rank order: benzoyl(*b*) ≈ *para*-hydroxy-*b* ≫ *para*-methoxy-*b* ≈ *para*-nitro-*b*. A putative explanation might be steric hindrance of the receptor interaction by *para*-substitution. In contrast, *ortho*-substitution of benzoyl resulted in an increased potency as compared to the lead with the exception of *ortho*-nitro. In particular, an *ortho*-fluoro substitution (AE 29, AE 49) was advantageous. Alkyl ketones (*type B compounds*) exhibited significantly stronger potency variations than the former and showed a clear size dependence of activity: the acetyl derivative AE 11 was the most potent and the cyclohexylcarbonyl analogue AE 18 was the weakest compound. The 6-substituents of *type C compounds* were not carbonyl-linked, but directly attached to the aromatic ring and exhibited strong chemical heterogeneity. Accordingly, a broad spectrum of vasorelaxant potency was observed, ranging from very weak (AE 45, EMD 54208) through intermediate (EMD 53704, AE 47) to rather high potency (AE 48, EMD 60893, bimakalim, EMD 55491).

3.4.2. Theoretical investigations

To understand the above described qualitative structure–activity relationships, we performed molecular modelling studies. LUMO energies, desolvation energies and molecular electrostatic potentials were calculated.

First, the possibility of charge transfer interactions was investigated. It was assumed that the electron-deficient aromate, being an electron acceptor, would bind to a corresponding electron donor within the receptor site. If charge transfer is important for receptor binding, small LUMO energies (Table 3) are expected when binding affinities are strong and vice versa. However, we found no correlation between these two parameters, as demonstrated by the plot in Fig. 8. The LUMO(benz) energy was smaller for AE 47 with intermediate potency than for the rather potent bimakalim, thereby contradicting, that charge transfer contributed to receptor binding.

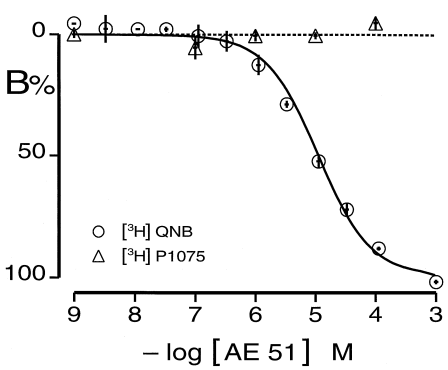

Fig. 7. Radioligand binding experiments with [^{3}H]QNB and [^{3}H]P1075 in disaggregated tracheal smooth muscle cells. Muscarinic acetylcholine receptors were radiolabelled with 0.44 nM [^{3}H]QNB and K_{ATP} channels with 1.62 nM [^{3}H]P1075. Increasing concentrations of AE 51 competed with [^{3}H]QNB (circles) but not with [^{3}H]P1075 (triangles) for binding. The dissociation constant pK_D ($-\log$, M) of AE 51 for muscarinic acetylcholine receptors estimated by non-linear regression according to Eq. (2) was 5.77 ± 0.04.

Table 3
LUMO(benz) energies and desolvation energies of benzopyrans with different substituents at position 6

Compound	LUMO(benz) energy (eV)	Desolvation energy (kcal/mol)
AE 9	−0.2569	−9.21
AE 11	−0.4871	−12.73
AE 15	−0.4817	−12.31
AE 18	−0.4806	−11.82
AE 20	−0.5287	−12.91
AE 21	−0.5253	−16.19
AE 22	−0.4898	−16.44
AE 23	−0.5085	−13.26
AE 25	−0.4837	−14.90
AE 26	−0.7516	−17.27
AE 29	−0.5652	−13.68
AE 30	−0.6031	−19.25
AE 33	−0.4890	−12.08
AE 34	−0.6724	−15.81
AE 44	−0.5408	−12.51
AE 45	−0.4018	−15.58
AE 47	−1.2262	−13.45
AE 48	−0.4757	−9.46
AE 49	−0.6219	−14.37
Bimakalim	−0.6119	−11.58
EMD 53704	−0.4415	−12.90
EMD 54208	−0.7916	−14.91
EMD 55491	−0.4785	−8.99
MD 60893	−0.6183	−10.82

The desolvation energies of the substituents can also contribute to binding (Table 3). This term comprises the amount of energy necessary to remove the water molecules from the surface of the ligand to facilitate receptor binding. Thus, the binding process is favored when this term is rather small; for example, more energy would be needed to desolvate AE 47 in comparison to bimakalim. After omission of AE 9 as outlier, there was a correlation between the pEC_{50}-values and the desolvation energies (see Fig. 8), underlining the importance of this term.

One of the most important forces in the recognition process between a receptor and a ligand is the electrostatic interaction. In Fig. 9, the molecular electrostatic potentials of bimakalim, AE 47 and AE 51 are presented in the plane of their benzopyran nucleus. The frontier potential lines close to the 6-substituent were contoured at a level of −60 kcal/mol in the case of bimakalim and AE 47, whereas for AE 51 the level was +100 kcal/mol. The degree of

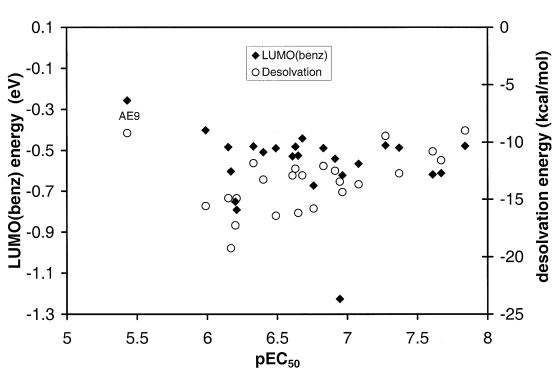

Fig. 8. The pEC_{50} values are plotted against the LUMO(benz) energy (rectangles) and against the desolvation energy (circles). The *Y*-axis on the left refers to the rectangles and the *Y*-axis on the right to the circles.

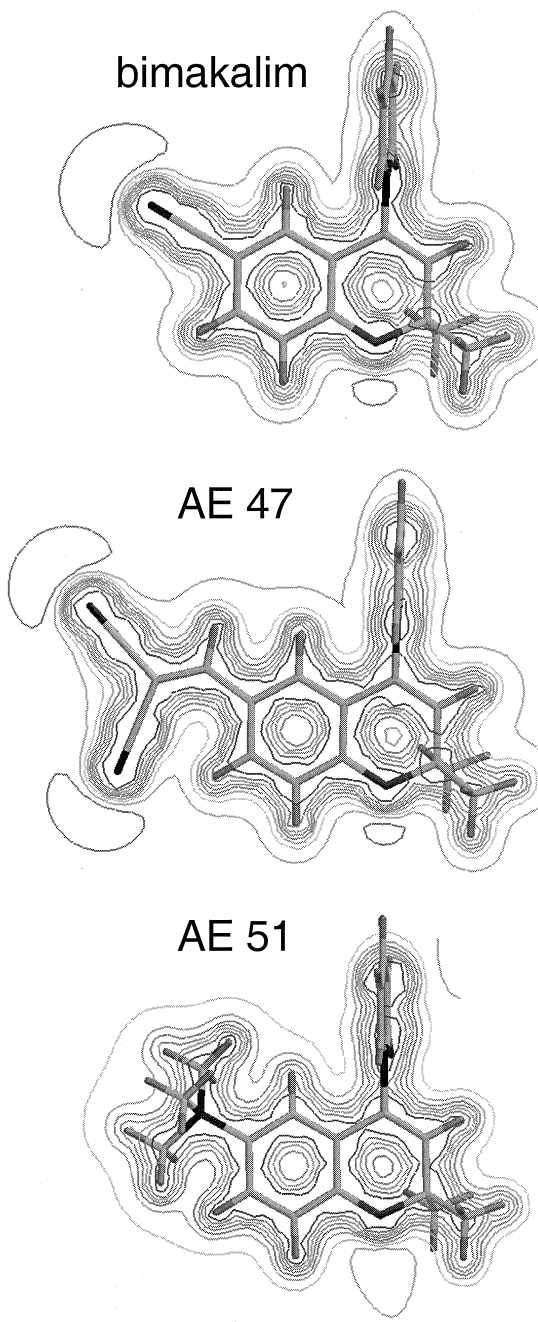

Fig. 9. The molecular electrostatic potentials of bimakalim, AE 47 and AE 51 are depicted in the plane of their benzopyran nucleus. The cap-like molecular electrostatic potential lines, located close to the cyano nitrogens of bimakalim and AE 47, are at a level of −60 kcal/mol. In contrast, the trimethylammonium group at the 6-position of AE 51 induces a positive molecular electrostatic potential, which wraps the whole molecule completely. The frontier contour shown is +100 kcal/mol.

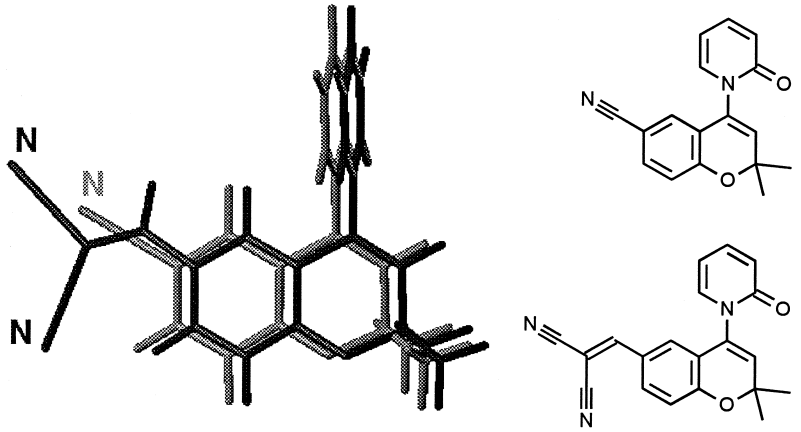

Fig. 10. Superposition of the geometry-optimized structure of bimakalim (grey) on that of AE 47 (black). The 2D-structures are shown on the right hand side.

congruence between the molecular electrostatic potentials of the individual compounds can indicate the importance of electrostatic interactions. The location of the maximum of the negative molecular electrostatic potentials close to the cyano nitrogens in AE 47 (Fig. 9) could not be fully superimposed with that in bimakalim, which was also true for the position of the cyano groups in the two molecules (Fig. 10).

The conversion of the biological effect of AE 51 from an agonist for K_{ATP} channels to a antagonist for muscarinic acetylcholine receptors can be understood in terms of the above data. If one accepts the view that a negative potential at the 6-position of the chromene nucleus is essential for binding to and opening of K_{ATP} channels, it is not surprising that the positively charged compound AE 51 exhibited a completely different pharmacological profile.

4. Discussion

4.1. Pharmacological properties

While ligands for a given G-protein-coupled receptor are chemically rather similar (e.g., β-adrenoceptor agonists and antagonists), ligands for some ion channels exhibit an extraordinary chemical diversity, indicating the existence of multiple binding sites on ion channels (e.g., L-type Ca^{2+}-channels) for chemically very different classes of drugs (Striessnig and Glossmann, 1992). ATP-sensitive K^+ channels are assembled with an octameric stoichiometry and are composed of a pore-forming α-subunit, an inward rectifying K^+ channel, and a regulatory β-subunit, the SUR, which carries the binding sites for channel-blocking sulfonylureas (e.g., glibenclamide, for review, see Aguilar-Bryan et al., 1998). SUR are members of the ATP-binding cassette protein superfamily, which is characterized by 13 membrane spanning helical regions and two nucleotide binding folds. Three isoforms have been cloned and are proposed to reconstitute the pancreatic β-cell (SUR1, Inagaki et al., 1995), the cardiac- (SUR2A, Inagaki et al., 1996), and the smooth muscle-type of K_{ATP} channel (SUR2B, Isomoto et al., 1996). In addition to binding sites for sulfonylureas, SUR also carry the binding sites for K_{ATP} channel activators (Hambrock et al., 1998; Schwanstecher et al., 1998). At least seven classes of chemically different structures interact with K_{ATP} channels followed by channel activation (Edwards and Weston, 1990).

Among the activators, the subgroup of benzopyrans is best characterized by pharmacological investigations and structure–activity analyses (Bergmann and Gericke, 1990; Gericke et al., 1991; Ishizawa et al., 1993; Koga et al., 1993; Ohta et al., 1994). The latter, however, are primarily focused on the 4-position of the benzopyran nucleus; our study was directed to an analysis of the importance of 6-substitutions. Rat aorta and trachea were selected as biological models because of the putative therapeutic application of K^+ channel activators in hypertension and asthma. As test set of compounds we used 24 benzopyrans with different substitutions at position 6, synthesized by Derix (1998). As compared to the unsubstituted lead, an adequate 6-substituent increased the relaxant potency by a factor of up to 500 both in aorta and trachea. Thus, K_{ATP} channel opening activity is sensitive to 6-substitution.

To prove whether the relaxant properties of the new benzopyrans are mediated via K_{ATP} channels, we performed binding studies with [^{3}H]P1075 with enzymatically disaggregated tracheal cells of the calf. The radioligand was a tritiated analogue of a cyanoguanidine that acts as K^+ channel opener (Manley et al., 1993) and which was originally characterized by its binding to intact smooth muscle rings of rat aorta with a dissociation constant of about 4 nM (Quast et al., 1993). [^{3}H]P1075 was used in competition binding studies to determine dissociation constants (K_D) for K^+ channel openers of different chemical classes. The K_D-values showed a good correlation with the EC_{50}-values for their relaxant action in functional experiments (rat aortic rings: Bray and Quast, 1992; calf coro-

nary arteries vs. cultivated coronary smooth muscle cells: Mannhold et al., 1996). Recently, it was shown (Lemoine et al., 1997) that [^{3}H]P1075 is also a suitable radioligand for K_{ATP} channels of tracheal smooth muscle, which is characterized by high affinity ($pK_D = 8.62 \pm 0.09$) and low receptor density (5750 ± 410 sites per cell), being approximately 50-fold lower than that of muscarinic acetylcholine receptors (Teschemacher et al., 1998). The high correlation of the pEC_{50} for relaxation with the pK_D for binding shows that the new compounds act via interaction with K_{ATP} channels and that the variation of relaxant potency of chemically different compounds is due to a variation of binding affinity and not due to a variation of their agonistic potency. Confirming observations from earlier investigations (Lemoine et al., 1996, 1997; Mannhold et al., 1996, 1999) we found weaker potencies for our benzopyrans in tracheal tissue than in aortic smooth muscle. Underlying explanations might be either differences in drug access to the receptor biophase or a reduced affinity of benzopyrans for K_{ATP} channels in the trachea. It appears unlikely that the difference in potency of the test compounds for vaso-vs. tracheo-relaxant effects via K_{ATP} channels was due to the use of different stimuli used for precontraction of tissues (carbachol in trachea vs. low 25 mM K^+ in aorta), as was shown by Cook et al. (1988) for cromakalim. These authors did not find that the use of different stimuli (angiotensin II, KCl, 5-hydroxytryptamine and noradrenaline) to precontract rabbit aorta influenced the estimates of EC_{50}s for relaxation by cromakalim.

As a probe for an intra- or extracellular location of the binding site for K^+ channel openers we designed and synthesized the permanently charged derivative AE 51, which contains a trimethylammonium moiety. It can be expected that AE 51 does not cross the cell membrane at least for the short duration of drug action (≈ 10 min, compare Fig. 4) under our experimental conditions. Surprisingly, the compound AE 51 was completely inactive in isolated aortic strips, whereas in tracheal strips a significant relaxation could be induced with micromolar doses of the compound (Fig. 4). Because it appeared unlikely that AE 51 exerted tissue selectivity in favor of the trachea, we took into account that the different stimuli used for contraction of aortic (25 mM K^+) and tracheal strips (0.6 μM carbachol) might explain the different ability of AE 51 to cause relaxation. Atropine, which binds with nanomolar affinity to muscarinic acetylcholine receptors of disaggregated tracheal cells (Teschemacher et al., 1998), antagonized the relaxant effects of AE 51 with high potency (Fig. 6), hinting at muscarinic receptors being involved in the mediation of the relaxant effects of AE 51. By radioligand binding, it was demonstrated that micromolar concentrations of AE 51 competed with a standard radioligand for muscarinic acetylcholine receptors (^{3}H-QNB, Fig. 7). A final proof for its antimuscarinic mode of action was found in functional experiments demonstrating simple competition with a muscarinic agonist (carbachol, Fig. 6): the basal tone of smooth muscle strips remained uninfluenced, concentration–effect curves for carbachol were shifted in a parallel manner, the blockade was surmounted by high carbachol concentrations and the Schild-plot analysis (Fig. 7, bottom) showed a slope of the regression line close to 1.0, indicating a simple competitive mode of antagonism. Like the radioligand binding experiments, the Schild-plot analysis yielded a micromolar dissociation constant for AE 51 at muscarinic acetylcholine receptors.

Which of the muscarinic acetylcholine receptor subtypes (M_1–M_4, Eglen et al., 1994) are involved in the mediation of the antimuscarinic effects of AE 51 remains unclear because each of the ligands used to compete with AE 51 (carbachol, atropine, [^{3}H]QNB) interacts with each receptor subtype. However, as a pure preparation of tracheal smooth muscle cells was used for the binding studies, it can be concluded that AE 51 at least binds to M_3 and M_2 receptors located on airway smooth muscle cells and antagonizes effects mediated by M_3 and M_2 receptors. M_3 and M_2 receptors are known to increase airway smooth muscle tone via stimulation of phosphatidylinositol turnover and the release of inositol-1,4,5-trisphosphate (IP_3) coupled to G_q-proteins (Chilvers and Nahorski, 1989) and via inhibition of β_2-adrenoceptor mediated stimulation of adenylyl cyclase coupled to G_i-proteins (Yang et al., 1991), respectively.

Obviously, the unexpected change in the pharmacological profile of AE 51, which turned out to act as muscarinic antagonist and not as agonist for K_{ATP} channels, prevented us from answering the initial question about the intra- or extracellular localization of the binding site of K_{ATP} channels for agonists.

4.2. Modelling investigations

Previous pharmacophore models for K^+ channel activators of the benzopyran type primarily reflect the importance of the 2-, 3- and 4-substitutions. Contradictory interpretations are found in the literature for the receptor interaction of the 6-substituent. According to Atwal (1994),

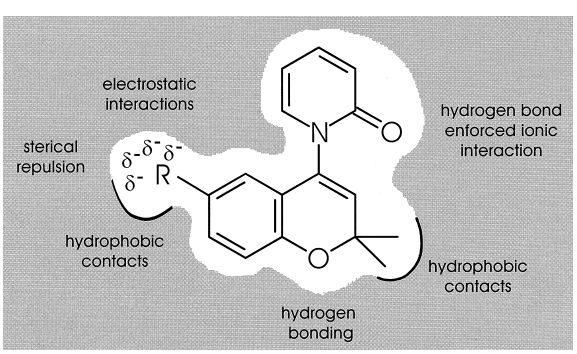

Fig. 11. Extended pharmacophore model for K^+ channel activators of the benzopyran-type with different substitution in the 6-position on the basis of the present data: the 6-substituent should be small and able to induce a negative electrostatic potential or should be polarizable; larger substituents will produce sterical repulsion.

the 6-substituent influences biological activity by withdrawing electrons from the aromatic part of the benzopyran nucleus. Thereby, the electron-deficient aromate could serve as a binding partner in charge transfer complexes.

The present data contradict this possibility. The results of the theoretical investigations clearly showed that a charge transfer interaction did not contribute to ligand-receptor binding, as would be indicated by an inverse relation between LUMO energies and binding affinities. We found that this correlation did not exist (Fig. 8). Thus, it is obvious that the respective 6-substituent itself must interact directly with the binding site and that its electron-withdrawing effect on the aromatic nucleus is of minor importance with respect to its binding affinity.

Taking into account all structure–activity data and the results of our theoretical investigations concerning the 6-substituent, we conclude that three qualities are necessary for high potency.

- Firstly, the substituent should be small in size.
- Secondly, with regard to the electronic properties, the 6-substituent either should be able to induce a strong negative molecular electrostatic potential close to the benzopyran nucleus (as is exemplified by bimakalim in Fig. 9 and also holds true for EMD 60893, AE 11 and AE 44) or should be polarizable, as is the case for the bromine substituent in EMD 55491.
- And, finally, it is advantageous, if the desolvation energy is small, because in such case a ligand will produce a larger energy gain by binding.

The scheme in Fig. 11 illustrates our current concept about a refined pharmacophore model for K^+ channel activators of the benzopyran type.

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References

Aguilar-Bryan, L., Clement, J.P., Gonzalez, G., Kunjilwar, K., Babenko, A., Bryan, J., 1998. Toward understanding the assembly and structure of K_{ATP} channels. Physiol. Rev. 78, 227–245.

Arunlakshana, O., Schild, H.O., 1959. Some quantitative uses of drug antagonists. Br. J. Parmacol. 14, 48–58.

Atwal, K., 1994. Pharmacology and structure–activity relationships for K_{ATP} modulators: tissue-selective K_{ATP} openers. J. Cardiovasc. Pharmacol. 24, S12–S17.

Bergmann, R., Gericke, R., 1990. Synthesis and antihypertensive activity of 4-(1,2-dihydro-2-oxo-1-pyridyl)-2*H*-1-benzopyprans and related compounds. New potassium channel activators. J. Med. Chem. 33, 492–504.

Blinks, J.R., 1965. Convenient apparatus for recording contractions of isolated muscle. J. Appl. Physiol. 20, 755–757.

Bray, K.M., Quast, U., 1992. A specific binding site for K^+ channel openers in rat aorta. J. Biol. Chem. 267, 11689–11692.

Chilvers, E.R., Nahorski, S.R., 1989. Phosphoinositide metabolism in airway smooth muscle. Am. Rev. Respir. Dis. 141, S137–S140.

Cook, N.S., Weir, S.W., Danzeisen, M.C., 1988. Anti-vasoconstrictor effects of the K^+ channel opener cromakalim on the rabbit aorta; comparison with the calcium antagonist isradipine. Br. J. Pharmacol. 95, 741–752.

Derix, A., 1998. 6-Substituierte Benzopyrane als Modulatoren des K_{ATP} Kanals. Synthese, Pharmakologie und Struktur-wirkungsbeziehungen. Thesis, Faculty of Science, Heinrich-Heine-Universität, Düsseldorf.

Edwards, G., Weston, A.H., 1990. Structure–activity relationships of K^+-channel openers. Trends Pharmacol. Sci. 11, 417–422.

Eglen, R.M., Reddy, H., Watson, N., Challiss, R.A.J., 1994. Muscarinic acetylcholine receptor subtypes in smooth muscle. Trends Pharmacol. Sci. 15, 114–119.

Ehle, B., Lemoine, H., Kaumann, A.J., 1985. Improved evaluation of binding of ligands to membranes containing several receptor-subtypes. Naunyn-Schmiedeberg's Arch. Pharmacol. 331, 52–59.

Ewertz, A., Lemoine, H., Weber, H., Mannhold, R., 1997. 6-Acyl-substituted benzopyrans as potassium channel openers (KCO): synthesis and pharmacological properties in aortic and tracheal preparations of the rat. Naunyn-Schmiedeberg's Arch. Pharmacol. 355, R272.

Gericke, R., Harting, J., Lues, I., Schittenhelm, C., 1991. 3-Methyl-2*H*-1-benzopyran potassium channel activators. J. Med. Chem. 34, 3074–3085.

Hambrock, H., Löffler-Walz, C., Kurachi, Y., Quast, U., 1998. Mg^{2+} and ATP dependence of K_{ATP}-channel modulator binding to the recombinant sulfonylurea receptor, SUR2B. Br. J. Pharmacol. 125, 577–583.

Hamilton, T.C., Weir, S.W., Weston, A.H., 1986. Comparison of the effects of cromakalim and verapamil on electrical and mechanical activity in rat portal vein. Br. J. Pharmacol. 88, 103–111.

Inagaki, N., Gonoi, T., Clement, J.P. IV, Namba, N., Inazawa, J., Gonzales, G., Aguilar-Bryan, L., Seino, S., Bryan, J., 1995. Reconstitution of I_{KATP}: an inward rectifier subunit plus the sulfonylurea receptor. Science 270, 1166–1170.

Inagaki, N., Gonoi, T., Clement, J.P. IV, Wang, C.Z., Aguilar-Bryan, L., Bryan, J., Seino, S., 1996. A family of sulfonylurea receptors determines the pharmacological properties of ATP-sensitive K^+ channels. Neuron 16, 1011–1017.

Ishizawa, T., Koga, H., Ohta, M., Sato, H., Makino, T., Kuromaru, K., Taka, N., Takahashi, T., Sato, T., Nabata, H., 1993. Structure–activity relationships of 6-substituted benzopyran-4-carbothioamide potassium channel openers. Bioorg. Med. Chem. Lett. 3, 1659–1662.

Isomoto, S., Kondo, C., Yamada, M., Matsumoto, S., Higashigushi, O., Horio, Y., Matsuzawa, Y., Kurachi, Y., 1996. A novel sulphonylurea receptor forms with BIR (Kir6.2) a smooth muscle type ATP-sensitive K^+ channel. J. Biol. Chem. 271, 24321–24324.

Koga, H., Ohta, M., Sato, H., Ishizawa, T., Nabata, H., 1993. Design of potent K^+ channel openers by pharmacophore model. Bioorg. Med. Chem. Lett. 3, 625–631.

Lemoine, H., 1992. β-Adrenoceptor ligands: characterization and quantification of drug effects. Quant. Struct.-Act. Relat. 11, 211–218.

Lemoine, H., Kaumann, A.J., 1983. A model for the interaction of competitive antagonists with two receptor-subtypes characterized by a Schild-plot with apparent slope unity. Agonist-dependent enantiomeric affinity ratios for bupranolol in tracheae but not in right atria of guinea pigs. Naunyn-Schmiedeberg's Arch. Pharmacol. 322, 111–120.

Lemoine, H., Ehle, B., Kaumann, A.J., 1985. Direct labelling of β_2-adrenoceptors. Comparison of binding potency of [^{3}H]ICI 118,551 and blocking potency of ICI 118,551. Naunyn-Schmiedeberg's Arch. Pharmacol. 331, 40–51.

Lemoine, H., Novotny, G.E.K., Kaumann, A.J., 1989. Neuronally released (−)-noradrenaline relaxes smooth muscle of calf trachea mainly through β_1-adrenoceptors: comparison with (−)-adrenaline and relation to adenylate cyclase stimulation. Naunyn-Schmiedeberg's Arch. Pharmacol. 339, 85–98.

Lemoine, H., Mannhold, R., Grittner, D., 1996. Binding studies with potassium channel openers (KCO) in smooth muscle cells of calf coronary artery as compared to rat cardiomyocytes. Naunyn-Schmiedeberg's Arch. Pharmacol. 353, R54.

Lemoine, H., Teschemacher, A., Mannhold, R., Divanac'h, A., 1997. Binding studies with potassium channel openers in intact smooth muscle cells of calf trachea. Naunyn-Schmiedeberg's Arch. Pharmacol. 355, R267.

Lemoine, H., Mannhold, R., Grittner, D., 1998. Restitution by nucleotides of the binding of the potassium channel activator [^{3}H]P1075 after metabolic inhibition in isolated rat heart cells. Naunyn-Schmiedeberg's Arch. Pharmacol. 357, R281.

Manley, P.W., Quast, U., Andres, H., Bray, K., 1993. Synthesis of and radioligand binding studies with a tritiated pinacidil analogue: receptor interactions of structurally different classes of K^+ channel openers and blockers. J. Med. Chem. 36, 2004–2010.

Mannhold, R., Lemoine, H., Jaspert, S., 1996. Comparison of binding and relaxant properties of potassium channel openers (KCO) in calf coronary arteries. Naunyn-Schmiedeberg's Arch. Pharmacol 353, R54.

Mannhold, R., Cruciani, G., Weber, H., Lemoine, H., Derix, A., Weichel, C., Clementi, M., 1999. 6-Substituted benzopyrans as potassium channel activators: synthesis, vasodilator properties and multivariate analysis. J. Med. Chem. 42, 981–991.

Ohta, M., Koga, H., Sato, H., Ishizawa, T., 1994. Comparative molecular field analysis of benzopyran-4-carbothioamide potassium channel openers. Bioorg. Med. Chem. Lett. 4, 2903–2906.

Quast, U., Bray, K.M., Andres, H., Manley, Y., Baumlin, Y., Dosogne, J., 1993. Binding of the K^+ channel opener [^{3}H]P1075 in rat isolated aorta: relationship to functional effects of openers and blockers. Mol. Pharmacol. 43, 474–481.

Schwanstecher, M., Sieverding, C., Dörschner, H., Gross, I., Aguilar-Bryan, L., Schwanstecher, C., Bryan, J., 1998. Potassium channel openers require ATP to bind to and act through sulfonylurea receptors. EMBO J. 17, 5529–5535.

Striessnig, J., Glossmann, H., 1992. L-type calcium channels and calcium channel ligands. In: Angeli, P., Gulini, U., Quaglia, W. (Eds.), Trends in Receptor Research. Pharmacochemistry Library, Vol. 18. Elsevier, pp. 333–343.

Teschemacher, A., Reinhardt, D., Lemoine, H., 1998. Do parasympatholytic effects of long-acting β_2-sympathomimetics contribute to their beneficial effects in the therapy of bronchial asthma?. Pulm. Pharmacol. Ther. 11, 253–261.

Yang, C.M., Chou, S.P., Sung, T.C., 1991. Muscarinic receptor subtypes coupled to generation of different second messengers in isolated tracheal smooth muscle cells. Br. J. Pharmacol. 104, 613–618.